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APPLICATION
FOR
UNITED STATES LETTERS PATENT

TITLE: PROMOTER SEQUENCES

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PROMOTER SEQUENCES

TECHNICAL FIELD

The present invention relates an isolated human Site-1 Protease promoter region. The 5 invention also relates to screening methods for agents decreasing the expression of Site-1 protease and thereby being potentially useful for the treatment of medical conditions related to obesity and / or diabetes.

10 BACKGROUND ART

Sterol Regulatory Element-Binding Proteins (SREBPs)

The integrity of cell membranes is maintained by a balance between the amount of 15 cholesterol and the amounts of unsaturated and saturated fatty acids in phospholipids. This balance is partly maintained by membrane-bound transcription factors called Sterol Regulatory Element-Binding Proteins (SREBPs; for reviews, see Brown & Goldstein (1997) Cell 89, 331-340; Brown & Goldstein (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 11041-11048) that activate genes encoding enzymes of cholesterol and fatty acid 20 biosynthesis. To enhance transcription, the active NH₂-terminal domains of SREBPs are released from endoplasmic reticulum membranes by two sequential cleavages. The first is catalyzed by Site-1 protease (S1P), a membrane-bound subtilisin-related serine protease that cleaves the hydrophilic loop of SREBP that projects into the endoplasmic reticulum lumen. The second cleavage, at Site-2, requires the action of S2P, a 25 hydrophobic protein that appears to be a zinc metalloprotease. These regulated proteolytic cleavage reactions are ultimately responsible for controlling the level of cholesterol in membranes, cells, and blood.

Three isoforms of SREBPs have been identified. SREBP-1a and SREBP-1c are encoded 30 by a single gene and differ in their N-terminal acid transcription activation domains. The N-terminus of SREBP-1a is longer and includes additional acidic amino acids, consistent with the observation that it is a more powerful transcription factor (Pai et al.

(1998) Proc. Natl. Acad. Sci. U.S.A. 40, 26138-26148). SREBP-2 is produced by a different gene and contains a long activation domain resembling that of SREBP-1a. Recent evidence suggests that the main function of SREBP-2 is to regulate cholesterol synthesis whilst that of SREBP-1 is to regulate fatty acid synthesis (Pai et al., *supra*).

5

Inhibition of SREBP transcription factor function will lead to reduced cellular synthesis of free fatty acids and cholesterol, the clinical benefits of which are expected to include increased cellular insulin sensitivity and reduced coronary artery disease (CAD).

10 Furthermore, SREBP-1 represents a cellular mechanism for increasing both fat cell size and number (Kim et al. (1998) J. Clin. Invest. 101, 1-9). Since most obesity generally involves an increase in both cell size and cell number, inhibition of SREBP-1 might be expected to have a positive effect on obesity. The hypolipidemic effects of dietary polyunsaturated fatty acids are believed to derive from a direct inhibitory effect on SREBP-1 expression (Xu et al. (1999) J. Biol. Chem. 274, 23577-23583).

15

There is data indicating independent regulation of SREBP-1 and SREBP-2 in hamster liver, suggesting the possibility for specific targeting of SREBP-1 or -2 (Sheng et al. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 935-938).

20 Transgenic mice over-expressing a dominant-positive form of SREBP-2 in the liver and adipose tissue showed greatly increased levels of mRNAs encoding multiple enzymes of cholesterol synthesis. Enzymes involved in fatty acid synthesis were also increased, however, to a lesser extent (Horton et al. (1998) J. Clin. Invest. 101, 2331-2339).

25 Transgenic mice over-expressing a constitutively active SREBP-1a in the liver and adipose tissue showed greatly increased mRNA levels for enzymes involved in fatty acid and cholesterol (Shimano et al. (1996) J. Clin. Invest. 98, 1575-1584). Their livers were enlarged about 4-fold due to a massive accumulation of free fatty acids and cholesterol. Over-expression of a corresponding version of SREBP-1c in adipocytes of transgenic mice yielded insulin resistance and diabetes (Shimomura et al. (1999) Genes Dev. 12, 3182-3194). In cell culture such overexpression was previously shown to promote adipocyte differentiation. It has further been shown that overnutrition increases SREBP-1c expression in liver and islets of obese fa/fa Zucker diabetic fatty rats (Kakuma, T. et al. (2000) Proc. Natl. Acad. Sci. U.S.A. 97: 8536-8541).

S-1 Protease

As discussed above, SREBPs are activated by proteolysis, which releases the active
5 transcription factor. The luminal subtilisin-like protease Site-1 Protease (S1P) is
responsible for the first of the two proteolytic steps. Cleavage by S1P enables further
cleavage by a Site-2 protease. S1P is the target for feedback inhibition by cholesterol.

S1P from hamster has been cloned (Sakai et al. (1998) Molecular Cell 2, 505-514).
10 (GenBank accession no. AF078105; SEQ ID NOS: 5 and 6). The corresponding
sequence of the (then unidentified) human gene was disclosed by Nagase et al. (1995)
DNA Research 2, 37-43 (GenBank Accession no. D420453; SEQ ID NOS: 3 and 4)

SREBP and S1P are co-localized with a third protein: SREBP Cleavage-Activating
15 Protein (SCAP), which is required for Site-1 cleavage *in vivo*. SCAP contains a site for
sterol regulation, conserved in a small number of proteins, e.g. HMG-CoA reductase.

Only one S1P has been identified among the human expressed sequence tags (ESTs).
Thus, S1P may be the only member of a subfamily among the subtilisin-like proteases.

20 Consequently, SREBPs are important regulators of fat and sugar metabolism in
mammals and direct or indirect down-regulation of SREBPs may be of therapeutic
value in type II diabetes; obesity, hypercholesterolemia, and other cardiovascular
diseases or dyslipidemias.

25 Site-1 Protease represents a molecular target for therapeutic intervention which is
expected to interfere with the SREBP pathway. Two principally distinct concepts for
inhibition of the site-1-protease activity may be postulated; (i) by inactivation of the
proteolytic activity (classical inhibitors) or (ii) by modulation of the site-1-protease gene
30 expression level. In order to modulate the expression of the site-1-protease gene, there is
a need for identification of regulatory regions responsible for the regulation of Site-1
protease promoter. Such regulatory regions in the promoter could be used for the

identification of agents that inhibit expression of Site-1 protease, and thereby for the inhibition of the SREBP pathway.

5 DISCLOSURE OF THE INVENTION

The 5'-flanking region (promoter region) of the human Site-1 Protease (S1P) gene has been cloned and sequenced. This promoter region is useful in biological assays for the identification of compounds that inhibit the transcription of the Site-1 Protease.
10 Inhibition of the SREBP pathway is expected to have therapeutic value in type II diabetes; obesity, hypercholesterolemia, and other cardiovascular diseases or dyslipidemias.

Consequently, in a first aspect this invention provides an isolated human site-1 protease 15 promoter region comprising a sequence selected from:

- (a) the nucleotide sequence set forth as SEQ ID NO: 2, or a fragment thereof exhibiting site-1 protease promoter activity;
- (b) the complementary strand of (a); and
- (c) nucleotide sequences capable of hybridizing, under stringent hybridization 20 conditions, to a nucleotide sequence as defined in (a) or (b).

The term "promoter region" refers to a region of DNA that functions to control the transcription of one or more genes, and is structurally identified by the presence of a binding site for DNA-dependent RNA polymerase and of other DNA sequences on the 25 same molecule which interact to regulate promoter function.

The nucleic acid molecules according to the present invention includes cDNA, chemically synthesized DNA, DNA isolated by PCR, genomic DNA, and combinations thereof. Genomic DNA may be obtained by screening a genomic library with the cDNA described 30 herein, using methods that are well known in the art.

In a preferred form of the invention, the said nucleic acid molecule has a nucleotide sequence identical with SEQ ID NO: 2 of the Sequence Listing. However, the nucleic

acid molecule according to the invention is not to be limited strictly to the sequence shown as SEQ ID NO: 2. Rather the invention encompasses nucleic acid molecules carrying modifications like substitutions, small deletions, insertions or inversions, which nevertheless have S1P promoter activity. Included in the invention are 5 consequently nucleic acid molecules, the nucleotide sequence of which is at least 90% homologous, preferably at least 95% homologous, with the nucleotide sequence shown as SEQ ID NO: 2 in the Sequence Listing.

The term "stringent hybridization conditions" is known in the art from standard 10 protocols (e.g. Ausubel et al., *supra*) and could be understood as e.g. hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at +65°C, and washing in 0.1xSSC / 0.1% SDS at +68°C.

The said "fragment" (partial sequence) exhibiting site-1 protease promoter 15 activity can be identified by the skilled person by computer-assisted sequence analysis, e.g. prediction of transcription factor binding sites.

The invention further provides a recombinant construct comprising the human site-1 protease promoter region as defined above. Preferably, the said construct comprises the 20 S1P promoter region operably linked to a gene encoding a detectable product, in particular the human site-1 protease gene (SEQ ID NO: 3).

The term "linked" indicates that a nucleotide sequence encoding a gene product and an 25 S1P promoter, or an active fragment thereof, are located within a continuous nucleic acid sequence. The term "operably linked" means that a nucleotide sequence, which can encode a gene product, is linked to the S1P promoter such that the S1P promoter regulates expression of the gene product under appropriate conditions. Two nucleotide sequences that are operably linked contain elements essential for transcription, including, for example, a TATA box.

30 The recombinant construct according to the invention could further comprise a reporter gene. As used herein, the term "reporter gene" means a gene encoding a gene product that can be identified using simple, inexpensive methods or reagents and that can be

operably linked to a S1P promoter or an active fragment thereof. Reporter genes such as, for example, a luciferase, β -galactosidase, alkaline phosphatase, or green fluorescent protein reporter gene, can be used to determine transcriptional activity in screening assays according to the invention (see, for example, Goeddel (ed.), *Methods Enzymol.*, 5 Vol. 185, San Diego: Academic Press, Inc. (1990); see also Sambrook, *supra*).

In another aspect the invention provides a vector comprising the recombinant construct as defined above. The term "vector" refers to any carrier of exogenous DNA that is useful for transferring the DNA to a host cell for replication and/or appropriate 10 expression of the exogenous DNA by the host cell. A host cell stably transformed with the recombinant construct is an additional aspect of the invention. Such a host cell can be a prokaryotic cell, a unicellular eukaryotic cell, or a cell derived from a multicellular organism. The methods employed to effect introduction of the vector into the host cell are standard methods well known to a person familiar with recombinant DNA methods. 15 The term "transformed" or "transfected" refers to the process by which exogenous DNA is transferred into an appropriate host cell.

In a further important aspect, this invention is useful in screening for pharmacological agents that modulate S1P levels by affecting the transcription of the S1P gene. As used 20 herein, the term "agent" means a biological or chemical compound such as a simple or complex organic molecule, a peptide, a protein or an oligonucleotide. Consequently, this invention includes a method for identifying an agent capable of modulating the S1P promoter, comprising providing a cell comprising the S1P promoter; contacting said cell with a candidate agent; and monitoring said cell for an effect that is not present in 25 the absence of said candidate agent.

A preferred form of the invention include a method for identification of an agent capable of decreasing or inhibiting site-1 protease promoter activity, said method comprising the steps (i) contacting a candidate agent with the human site-1 protease 30 promoter; and (ii) determining whether said candidate agent decreases expression of the site-1 protease gene, such decrease being indicative for an agent capable of decreasing or inhibiting site-1 protease promoter activity.

For screening purposes, appropriate host cells can be transformed with a vector having a reporter gene under the control of the human S1P promoter according to this invention. The expression of the reporter gene can be measured in the presence or absence of an agent with known activity (i.e. a standard agent) or putative activity (i.e. a "test agent" 5 or "candidate agent"). A change in the level of expression of the reporter gene in the presence of the test agent is compared with that effected by the standard agent. In this way, active agents are identified and their relative potency in this assay determined.

It will be understood that agents acting on the human S1P promoter can be identified 10 by, as an additional step, analyzing direct binding interactions between the candidate agent and the human S1P promoter. Interactions with large molecules may be studied using techniques such as gel shift analysis, footprinting or NMR (see Latchman, D.S. (Ed.) (1995) Methods for studying transcription factors. In: Eukaryotic transcription factors. Academic Press, London, pp. 17-44). Small molecule compounds which appear 15 to bind reversibly to double stranded DNA without intercalation between DNA base pairs have been defined. Methods are described by which this non-intercalative binding can be characterized using ultraviolet spectrometry, fluorimetry with ethidium as a probe, viscometry and other hydrodynamic techniques, circular dichroism and nuclear magnetic resonance spectrometry (See Baguley, B.C. (1982) Nonintercalative DNA- 20 binding antitumour compounds. Mol Cell Biochem 43: 167-181; Gmeiner, W.H. (1998) NMR spectroscopy as a tool to investigate the structural basis of anticancer drugs. Curr Med Chem 5(2):115-135; Wemmer, D.E. & Williams, P.G. (1994) Use of nuclear magnetic resonance in probing ligand-macromolecule interactions. Methods Enzymol. 239:739-767)

25 A potentially useful method for identification of agents acting on the human S1P promoter is described in Swedish patent application No. 0101218-6, filed on 5 April 2001. Such a method comprises the steps

- (a) predicting the structure of an RNA-fragment;
- (b) choosing a suitable predicted RNA-fragment of step (a), which RNA-fragment comprises at least one individual stem;
- (c) synthesizing the DNA-fragment corresponding to the RNA-fragment of step (b);

(d) inserting the DNA-fragment of step (c) in the upstream proximity of a reporter assay gene, which reporter assay gene produces a signal upon translation, thereby forming a reporter construct;

(e) performing a reporter gene assay, which assay monitors the interaction between a 5 molecule to be tested for RNA-binding and the RNA-fragment of the reporter construct.

As mentioned above, it is expected that agents capable of decreasing or inhibiting site-1 protease promoter activity have potential therapeutic value in particular in obesity, and in type II diabetes; hypercholesterolemia, atherosclerosis and other cardiovascular 10 diseases or dyslipidemias. Consequently, the invention comprises a method for the treatment of medical conditions related to obesity, comprising administering to a patient in need thereof a therapeutically effective amount of an agent identified by the method according to the invention.

15 The term "treatment" means any treatment of a disease in a mammal, including: (i) preventing the disease, i.e. causing the clinical symptoms of the disease not to develop; (ii) inhibiting the disease, i.e. arresting the development of clinical symptoms; and/or (iii) relieving the disease, i.e. causing the regression of clinical symptoms. The term "effective amount" means a dosage sufficient to provide treatment for the disease state 20 being treated. This will vary depending on the patient, the disease and the treatment being effected.

Throughout this description the terms "standard protocols" and "standard procedures", when used in the context of molecular biology techniques, are to be understood as 25 protocols and procedures found in an ordinary laboratory manual such as: Current Protocols in Molecular Biology, editors F. Ausubel et al., John Wiley and Sons, Inc. 1994, or Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989.

EXAMPLES

EXAMPLE 1: Cloning of S1P promoter fragment

5 For cloning of the 5'-flanking region of the S1-protease gene, a genomic walking strategy was used, principally as described by Siebert et al. (1995) Nucleic Acids Res. 23,1087-1088; and Siebert et al. (1995) CLONTECHniques X, 1-3. Two primers, designated FOMA 345 and FOMA 346, were selected in the 5'-region of the cDNA: FOMA 345: 5'-CTC CGC GGC GAA CAC GCCT-3' (corresponding to positions 126-
10 108 in SEQ ID NO: 3);
FOMA 346: 5'-CGG GAG CTC AGG GCC GGC-3' (corresponding to positions 163-146 in SEQ ID NO: 3).

All other reagents used were obtained with a "Genome Walker Kit" (Clontech, Palo Alto, CA). The principle of this procedure is to perform two subsequent PCR reactions using adaptor-ligated genomic DNA as template. In the first PCR reaction the "outer" primers are used, i.e. FOMA 345 and AP1 (adaptor primer 1). The protocol for this reaction was:

(+95° for 25 sec; +72° for 4 min) x 7 cycles (+95° for 25 sec; +67° for 4 min) x 35 cycles (+67° for 4 min) x 1 cycle

20 In the second PCR reaction, the "inner" primers were used (FOMA 346 and AP2). The reaction mix from the first PCR was diluted 50 times and 1 µl of this cocktail was used as template in the second reaction. The protocol of the second reaction was:
(+95° for 25 sec; +72° for 4 min) x 5 cycles
25 (+95° for 25 sec; +67° for 4 min) x 25 cycles
(+67° for 4 min) x 1 cycle

30 The reaction mixes were prepared in accordance with the instructions of the kit manufacturer. After the second PCR, the product was analyzed by electrophoresis in 2% agarose gel. A product, approximately 1 kb long, was observed in one of the adaptor-ligated genomic DNA-libraries (HDL2). This product was cloned into the TOPO vector PCR2.1 (Invitrogen, Carlsbad, CA) by standard cloning procedures and thereafter sequenced. A 980 bp sequence was obtained (SEQ ID NO: 1).

EXAMPLE 2: Assembly of S1P promoter sequence

5 The Celera database (Release 1.13) was searched using the 980 bp sequence obtained in Example 1 as query sequence. The BLAST algorithm (Altschul et al. (1997) Nucleic Acids Res. 25:3389-3402) was used for determining sequence identity. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). Six fragments
10 (GA_16330554; GA_25791426; GA_23194195; GA_28969362; GA_18902492; GA_24454650) that overlapped with the genomic sequence were retrieved. These six fragments were used to search the Celera database (Release 1.13) again, the overlapping sequences were extended with another 7 fragments (GA_24421404, GA_21984802, GA_28735370, GA_21045430, GA_9491232, GA_13453697, GA_25224137). All 13
15 fragments together with the 980 bp sequence obtained in Example 1 were finally assembled to a 2469 bp contig (SEQ ID NO: 2) using the Cap2 program (Huang (1996) Genomics 33, 21-31).

20 EXAMPLE 3: Reporter gene assay to identify modulating compounds

Reporter gene assays are well known as tools to signal transcriptional activity in cells. (For a review of chemiluminescent and bioluminescent reporter gene assays, see Bronstein et al. (1994) Analytical Biochemistry 219, 169-181.) For instance, the
25 photoprotein luciferase provides a useful tool for assaying for modulators of S1P promoter activity. Cells (e.g. CHO cells or COS 7 cells) are transiently co-transfected with both a Site-1 protease expression construct and a reporter construct which includes a gene for the luciferase protein downstream from a transcription factor binding site. Luciferase activity may be quantitatively measured using e.g. luciferase assay reagents
30 that are commercially available from Promega (Madison, WI). Differences in luminescence in the presence versus the absence of a candidate modulator compound are indicative of modulatory activity.

A luciferase reporter plasmid is prepared by cloning a 980 bp sequence (SEQ ID NO: 1) corresponding to a part of the site-1-protease promoter into the pGL2 vector, in which the luciferase reporter gene is driven by the activity of the inserted promoter. The construct is thereafter transfected into the mouse pre-adipocyte cell line 3T3-L1 (ATCC 5 No. CCL92.1), the human embryonic kidney cell line 293 (ATCC No. CRL-1573), and the human hepatoma cell line HepG2 (ATCC No. HB-8065). Altered promoter activity after stimulation with a number of substances, including insulin, glitazones and sterols, are measured as changes in the readout of luciferase.